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Triterpenes and Cytotoxic Activity of *Acokanthera oblongifolia* Hochst. Growing in Egypt

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ABSTRACT

Five known triterpenes, α-amyrin, (1), lupeol acetate (2), betulinaldehyde (3), lupeol (4), and betulinic acid (5) were isolated from *Acokanthera oblongifolia* leaves. Their structures were identified on the basis of spectroscopic analysis, including ^{1}H, ^{13}C NMR and MS. The *in-vitro* cytotoxicity of the total chloroformic extract and the main isolated triterpene compound (α-amyrin) was evaluated against three different human cell lines, hepatocellular carcinoma (HepG2), breast adenocarcinoma (MCF7) and colon cell line (HCT116). The chloroformic extract showed a moderate cytotoxic activity with IC_{50}= 37.6, 65.4 and 66.8 µg/ml, respectively.

Keywords: *Acokanthera oblongifolia, Apocynaceae, Triterpenes, Cytotoxic activity.*

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INTRODUCTION

Acokanthera oblongifolia Hochst. (Synonyms *Acokanthera spectabilis, Carissa oblongifolia, Toxicophloea spectabilis)* common names (Bushmann's poison, poison bush, poison tree, wintersweet) belongs to family apocynaceae. This is a large family including about 1500 species in 180 genera, most of them have an economic and medicinal importance. Many species are found in the tropics and few are growing in common temperate regions. This plant is considered an ornamental poisonous one (Morton, 1958, 1962, 1971, 1977). It can be used medicinally to treat snakebites and as emetic by eating a small amounts of the leaves. The leaf or wood decoction is used also in the treatment of internal worms. Not only, the unripe fruits are highly toxic but also, the other different parts of the plant are toxic (Schmelzer & Agurib-Fakim, 2008). Ingestion of this plant may cause lethargy, restlessness and seizures. Related species in other parts of the world have been reported as causing deaths. Eating the fruit can cause severe gastro-intestinal irritation with abdominal pain, excessive salivation and vomiting. The toxicity of the fruit appears to be variable and the sap is an irritant to the skin and eyes (Kupicha 1982, Neuwinger, 2000). Numerous cardenolides were detected in the wood, leaves, seeds and fruits of *Acokanthera spectabilis*. The most important being acovenosides A and B, which both have cardio-vascular properties. Other cardenolides that were isolated are acovenoside C, spectabiline and acopieroside II. The last compound shows a higher activity than digitalin and digoxin as a cardiotonic. The seeds yielded acobioside A and 14-O-acetyl-acovenoside C which showed a marked cardiotonic activity in dogs (Hanna et al. 1998, Neuwinger 1996, Karawya et al. 1974, Schlegel, et al. 1955 and De Villers, 1962). According to Watt & Breyer-Brandwijk (1962), an ointment made from the fine scrapings of the root of *Acokanthera spectabilis* is used by the Mpondo for the relief of itchy conditions. The application is said to be violently irritant Gardner & Bennetts (1956) including *Acokanthera spectabilis* in a list of plants known or suspected of causing urticaria or skin irritation. Grant (1974) and Morton (1977) noted that, the contact with *Acokanthera spectabilis* may cause smarting of the eyes and skin, and throat irritation.

In this study, we are going to investigate the main constituents of the chloroform extract of *Acokanthera oblongifolia* plant along with the cytotoxicity of the chloroform extract and the main isolated components against three different human cell lines, human hepatocellular carcinoma (HepG2), human breast adenocarcinoma (MCF7) and colon cell line (HCT116).

MATERIAL AND METHODS

Plant material:

The leaves of *Acokanthera oblongifolia* were collected from Alnobaryia region, Egypt during May 2012, and kindly identified with Prof.Dr. Kamal Zayed professor of Botany, Cairo University, Cairo, Egypt. A voucher specimen was kept in the Herbarium of, National Research Centre, Cairo, Egypt. The plant was air dried and grinded to a fine powder.

General experimental procedures:

TLC was carried out on precoated silica gel F_{254} plates (Merck) (Darmstadt, Germany) developed with *n*-hexane:EtOAc (90/10), (80/20) and (70/30). Spots were detected by examining the chromatoplates in the UV light at 366 nm and 254 nm before and after spraying with vanillin/sulphuric acid reagent (5% H_2SO_4 and 1% vanillin in methanol solution) mixed just before use. Column chromatography was performed on silica gel 70-230 mesh (Merck) and Sephadex LH-20 (Pharmacia). Mass spectra were recorded on a JEOL-JMS-AX 500 Mass spectrometer (EI-MS) and (FAB-MS) using glycerol as liquid matrix. ^{1}H-NMR was recorded in $CDCl_3$, on Jeol- GX at 500 MHz. using TMS as internal standard.

Phytochemical investigation

Extraction and isolation:

Acokanthera oblongifolia leaves (750 g) were extracted with $CHCl_3$ (5 × 1 L) at room temperature. The extracts were combined and the solvent were removed under vacuum to yield 62 g. The $CHCl_3$ extract was dissolved in 400 ml of $CHCl_3$-MeOH (1:1), and then mixed with 30 g charcoal at 40 ^oC for removing the chlorophyll pigments, then filtered. The filtrate was evaporated to dryness under vacuum. The final $CHCl_3$ extract (12.5 g) was subjected to silica gel column chromatography (120 × 3 cm) and eluted with mixtures of *n*-hexane and EtOAc of increasing polarity and monitored by TLC. Twelve fractions (FR. 1-12) were obtained. Fraction 3 (755 mg) which was eluted with (*n*-Hexane-EtOAc, 95:5), was rechromatographed on another silica gel column (90 × 1.5 cm) eluted with toluene-$CHCl_3$ in gradient). Subfraction which was eluted with toluene-$CHCl_3$ (70:30) yielded compound 1 (58 mg). While, compound 2 (26 mg) was obtained from subfraction eluted with toluene-$CHCl_3$ (50:50). Rechromatography of FR. 5 which was eluted from the first column by *n*-hexane-EtOAc (9:1) on a sephadex column chromatography (70 × 2 cm) eluted with methylene chloride-methanol (1:1) followed by third silica gel column chromatography eluted with toluene-$CHCl_3$ with increasing polarity to yield 6 subfractions (A-F). Subfraction B was subjected to preparative TLC using silica gel coated aluminium sheets 0.5 mm thickness running with toluene-$CHCl_3$ (8:2) to yield compound 3 (9 mg). While, compound 4 (16 mg) was obtained as a colorless powder from subfraction E. Also, compound 5 (32 mg) was obtained as a white amorphous powder from subfraction F.

Cytotoxic Activity

1. Cell Culture and Sample Treatment:

The cell lines under investigation were human breast adenocarcinoma (MCF7), human hepatocellular carcinoma (HepG2), human lung carcinoma (A549) and human colon cell line (HCT116). They were purchased from American Tissue Culture Collection. HepG2, MCF7 and HCT116 cells lines were cultured in RPMI 1640 medium while A549 cell line was cultured in DMEM medium. Media are supplemented with 1% antibiotic–antimycotic mixture (10,000 UmL^{-1} potassium penicillin, 10,000 mg mL^{-1} streptomycin sulphate and 25 mg mL^{-1} amphotericin B), 1% L-glutamine and 10% fetal bovine serum. According to the cells

growth profile, cells were seeded with a density of 1×10^4 cell per well. This number was sufficient to give a reliable reading with the MTT assay), which corresponded well with the cell number and was the one that gave exponential growth throughout the incubation period with the tested sample (Mosmann, 1983).

2. *In vitro* Cytotoxic Activity:

The antiproliferative activity of tested samples were measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide "Promega, USA") assay. The assay detects the reduction of MTT by mitochondrial dehydrogenase to blue formazan product, which reflects the normal function of mitochondria and cell viability. Exponentially growing cells were washed and seeded at 1×10^4 cells per well (in 200 µL of growth medium) in 96 well microplates. After 24 h incubation, a partial monolayer was formed then the media was removed and 100 µL of the medium containing different concentrations of tested samples (initially dissolved in DMSO) were added and re-incubated for 48 h. Then, 100 µL of the medium were aspirated and 40 µL of the MTT solution were added in each well. After 4 h contact with the MTT solution, blue crystals were formed. 150 µL of the SDS stop solution were added and further incubated for 1 h. Reduced MTT was assayed at 595nm using a microplate reader (Thabrew et al., 1997). Control groups received the same amount of DMSO (0.1%). Untreated cells were used as a negative control while cells treated with doxorubicin were used as a positive control at a concentration of 100 µg mL^{-1}. Stock solutions of the plant extract were dissolved in DMSO then diluted with the medium and sterilized using 0.2 µm membrane filters. The final dilution of extracts used for treating the cells contained no more than 0.1% DMSO. The percentage of change in viability was calculated according to the formula [1-(reading of extract/reading of negative control)] ×100. A probit analysis was carried out for IC_{50} and IC_{90} estimation using SPSS 11 program.

RESULTS AND DISCUSSION

The $CHCl_3$ extract of *Acokanthera oblongifolia* leaves was chromatographed over silica gel and eluted with *n*-hexane and EtOAc to yield 5 triterpene compounds.

Compound 1: white powder; EI-MS 70 eV *m/z* 426 [M^+] ($C_{30}H_{50}O$). ^1H and ^{13}C NMR ($CDCl_3$) spectral data are shown in Tables 1 and 2. From comparison of these spectral data with the literature, compound 1 was identified as α-amyrin (Lima et al., 2004; Dias et al., 2011).

Compound 2: yellow solid; EI-MS 70 eV *m/z* 468 [M^+] ($C_{32}H_{52}O_2$). ^1H and ^{13}C NMR ($CDCl_3$) are shown in Tables 1 and 2. MS, ^1H and ^{13}C NMR spectral data were in agreement with lupeol acetate (Prachayasittikul et al., 2010).

Compound 3: white powder; EI-MS 70 eV *m/z* 440 [M^+] ($C_{30}H_{48}O_2$). ^1H and ^{13}C NMR ($CDCl_3$) are shown in Tables 1 and 2. The spectral data of this compound were identical with betulinaldehyde (Theerachayanan et al., 2007).

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Table 1. ^{1}H NMR spectral data of the isolated compounds (1-5).

Position	Compound				
	1	**2**	**3**	**4**	**5**
3	3.17 (*dd*, *J*= 5.1, 11.2)	4.45 (*dd*, *J*= 10.8)	3.3 *q*	3.16 (*dd*, *J*= 10.8, 5.5)	3.79 *s*
5	0.67 (*d*, *J*= 11.6)	0.76 (*dd*, *J*= 10.8, 5.8)		0.66 (*d*, *J*= 9.1)	
12	5.06 (*t*, *J*= 3.2)				
15	1.95 (*td*, *J*= 4.5, 13.5)				
16	1.76 (*td*, *J*= 5.0, 13.5)				
19		2.33 (*dt*, *J*= 11.1, 5.6)	2.80 *m*	2.36 (*dt*, *J*= 10.9, 5.5)	2.39
21		1.86 *m*		1.89 *m*	
22	1.85 (*dt*, *J*= 3.0, 7.0)				
23	0.93 *s*	0.82 *s*	0.84 *s*	0.94 *s*	0.94
24	0.74 *s*	0.82 *s*	0.68 *s*	0.73 *s*	0.80
25	0.73 *s*	0.82 *s*	0.75 *s*	0.80 *s*	0.74
26	0.89 *s*	1.00 *s*	0.90 *s*	1.00 *s*	0.97
27	1.01 *s*	0.91 *s*	1.19 *s*	0.92 *s*	1.00
28	0.94 *s*	0.81 *s*	9.6 *s*	0.76 *s*	
29	0.85 (*d*, *J*= 6.0)	4.54 *br s*	4.56, 4.69 (*d*, *J*= 2.0)	4.58 (*br s*)	4.66
30	0.73 (*d*, *J*= 7.0)	1.67 *s*	1.62 *s*	1.34 *s*	1.66
2`		2.01 *s*			

Compound 1=α-amyrin, Compound 2=lupeol acetate, Compound 3=betulinaldehyde, Compound4=lupeol, Compound5=betulinic acid

Compound 4: white powder; EI-MS 70 eV *m/z* 426 [M^{+}] ($C_{30}H_{50}O$). ^{1}H and ^{13}C NMR ($CDCl_3$) are shown in Tables 1 and 2. Comparison of these data with literature models led us to identify this compound as lupeol (Prakash and Prakash, 2012).

Compound 5: white amorphous powder; EI-MS 70 eV *m/z* 456 [M^{+}] ($C_{30}H_{48}O_3$). ^{1}H and ^{13}C NMR ($CDCl_3$) are shown in Tables 1 and 2. From MS, ^{1}H and ^{13}C NMR spectral data, compound 3 were identified as betulinic acid (Uddin et al., 2011).

All of these compounds are isolated from *Acokanthera oblongifolia* leaves for the first time. While, there are many triterpene compounds isolated from different plants species belonging to family apocynaceae. β-amyrin acetate and lupeol were isolated from *Alstonia boonei* (Faparusi and Bassir, 1972). From *parsonsia laevigata,* taraxerol and lupeol were identified (Ogihara, et al. 1987). Also, oleanderolic acid and anerodione were detected in *Nerium oleander* (Siddiqui, et al. 1989).

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Table 2. ^{13}C NMR spectral data of the isolated compounds (1-5).

Position	Compound				
	1	2	3	4	5
1	38.7	38.3	38.6	38.0	38.8
2	27.1	23.6	27.3	25.2	27.9
3	78.3	80.9	78.9	78.4	79.0
4	38.7	37.7	38.8	38.5	38.7
5	55.2	55.3	55.2	55.1	55.6
6	18.3	18.2	18.2	18.1	18.3
7	32.9	34.2	34.3	34.2	34.3
8	40.1	40.8	40.8	41.1	40.9
9	47.7	50.3	50.4	49.7	50.5
10	36.9	37.0	37.1	73.3	37.2
11	23.3	20.9	20.7	21.1	20.8
12	124.2	25.1	25.5	27.5	25.2
13	139.3	38.0	38.7	39.2	38.4
14	42.0	42.8	42.5	42.6	42.4
15	28.7	27.4	29.2	27.5	30.6
16	26.6	35.5	28.8	35.6	32.1
17	33.7	42.9	59.4	43.2	56.3
18	58.9	48.0	48.1	48.2	46.8
19	39.6	48.2	47.5	47.8	49.2
20	39.6	150.9	149.7	151.6	150.4
21	31.2	29.8	29.8	30.1	29.8
22	41.5	39.9	33.3	40.2	34.2
23	28.1	28.2	27.9	28.2	28.0
24	15.6	15.9	15.3	16.0	15.3
25	15.6	16.1	15.8	16.8	16.0
26	16.8	16.3	16.1	16.4	16.1
27	23.3	14.5	14.2	15.1	14.8
28	28.1	17.9	206.7	18.1	180.3
29	17.4	109.2	110.2	108.5	109.6
30	21.2	19.0	19.0	19.5	19.5
C 1`		171.0			
C 2`		21.3			

Compound 1=α-amyrin, Compound 2=lupeol acetate, Compound 3=betulinaldehyde, Compound4=lupeol, Compound5=betulinic acid.

Cytotoxic activity:

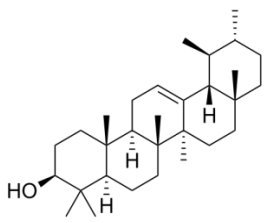
As shown in table 3, the *in-vitro* cytotoxicity of the total chloroformic extract and the main isolated triterpene compound (α-amyrin) was evaluated against three different human cell lines, hepatocellular carcinoma (HepG2), breast adenocarcinoma (MCF7) and colon cell line (HCT116) with the comparison of doxirubacin as a positive control. The chloroformic extract showed a moderate cytotoxic activity with IC_{50}= 37.6, 65.4 and 66.8 µg/ml, respectively. This activity may be attributed to the presence of lupeol and betulinic acid since the recent reports showed that triterpenes directly inhibit tumor growth cell cycle progression, and induce the apoptosis of tumor cells under *in vitro* and *in vivo* situation. While α-amyrin was found to be inactive as shown in table 3. Lin et al. (2011) stated that β-amyrin exhibited weak cytotoxic activities against human bladder cancer cells (NTUB1). Also, β-amyrin showed no cytotoxic activities against cervix adenocarcinoma (Hela), breast

adenocarcinoma (MCF7) and skin epidermoid carcinoma (A431) cells (Csapi et al., 2010). Our results are found to be in agreement with that obtained by (Saleem, 2009) who reported that betulinic acid which is a natural product with a range of biological effects, for example potent antitumor activity. This anticancer property is linked to its ability to induce apoptotic cell death in cancer cells by triggering the mitochondrial pathway of apoptosis (Fulda, 2008). Lupeol and lupeol acetate showed a cytotoxic activity against MCF7 cells at IC_{50}= 17 and 26 µg/ml, respectively (Lee Shan et al., 2014). Archana et al. (2013) stated that betulinic acid showed *in vitro* cytotoxicity towords MCF7 cells with IC_{50} value of 13.5 µg/ml. Neto (2007) reported that there is an anti-proliferative activity of urosolic acids in wide varieties of cancer cell lines. The triterpene esters inhibited the growth of lung, colon, breast and renal cancers (Kondo, 2006). Complementary effects of the triterpene compounds are likely to play a role in decreased tumor cell proliferation (Liberty et al., 2009).The presence of lupeol, lupeol acetate and betulinic acid explained the cytotoxic activity of the total chloroformic extract of *Acocanthera oblongifolia*. This result encourages the authors to continue their work to test the isolated compounds against other cell lines to find out more biological activities of this plant.

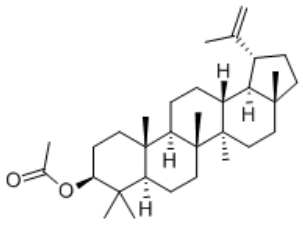
Table 3. The cytotoxic activity of *Acokanthera oblongifolia* leaves against several types of cancer cell lines.

Compound	IC_{50} (µg/ ml)		
	MCF7	HCT116	HepG2
$CHCl_3$ extract	65.4	66.8	37.6
α-amyrin	≤100	≤100	≤100
Doxirubacin	26.1	37.6	21.6

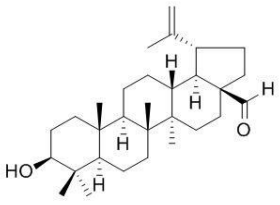
IC_{50}: dose of the drug which reduces survival cells to



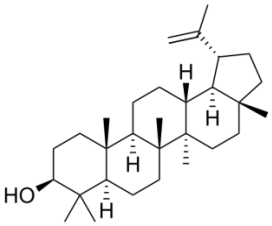
compound 1: α-amyrin



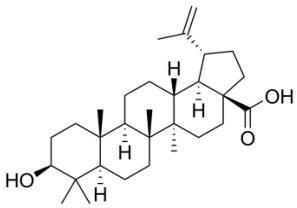
compound 2: lupeol acetate



compound 3: betulinaldehyde



compound 4: lupeol



compound 5: betulinic acid

Fig.1. Structures of isolated compounds (1-5).

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